Current- and Voltage-Clamped Studies on *Myxicola* Giant Axons

Effect of tetrodotoxin

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ABSTRACT   A new dissection procedure for preparing *Myxicola* giant axons for observation under voltage clamp is described. Preparation time is generally 40–45 min. 65–70 % of the preparations attempted may be brought through the entire procedure, including insertion of the long internal electrode, and support an initial action potential amplitude of 100 mv or greater. Mean values for axon diameter, resting membrane potential, action potential amplitude, maximum peak inward transient current, and resting membrane resistance are 560 μ, −66.5 mv, 112 mv, 0.87 ma/cm², and 1.22 KΩ cm², respectively. Cut branches do not seem to be a problem in this preparation. Behavior under voltage clamp is reasonably stable over several hours. Reductions in maximum inward transient current of 10 % and in steady-state current of 5–10 % are expected in the absence of any particular treatment. Tetrodotoxin blocks the action potential and both the inward and outward transient current, but has no effect on either the resting membrane potential or the steady-state current. This selective action of tetrodotoxin on the transient current is taken as an indication that this current component is probably carried by Na.

INTRODUCTION

Certainly one of the most powerful methodologies brought to the study of excitable membranes is the space voltage clamp (Cole, 1949; Hodgkin et al., 1952). Two desirable criteria that any application of this technique should satisfy to be most useful for quantitative studies are that it permit a reliable determination of the absolute membrane potential and that it provide reasonably stable responses over a period of time. For nervous tissue these criteria have been well-satisfied only for the sort of technique applied to squid giant axons, which may be studied using long internal current-passing and potential-sensing electrodes. Node and artificial node preparations although useful in

730

other respects do not well satisfy these criteria (Dodge and Frankenhaeuser, 1958; Blaustein and Goldman, 1966).

Unfortunately, squid are usually available only seasonally and do not survive well in the laboratory. However, another preparation, the giant axon of *Myxicola*, can also accommodate insertion of long internal electrodes or cannulae and can be made available in the laboratory the year around. *Myxicola* is expected, then, to be an important preparation for the study of membrane processes because of the expanded effort that it will permit.

For *Myxicola* to be as useful a preparation as the squid has been for the study of membrane phenomena, it needs to be as well-understood quantitatively. This series of papers begins the systematic development of *Myxicola* as a routine laboratory preparation. This first paper describes a greatly improved preparation procedure as well as summarizing the now extensive, current and voltage-clamp data on base line values, stability, and drug effects.

METHODS

Myxicola infundibulum (Polychaeta) were obtained from a commercial supply house (Maritime Biological Laboratories, St. Stephen, New Brunswick). In an approximately 2 yr period over which regular shipments were received, there were no particular seasonal fluctuations in size or abundance. Animals maintained in the laboratory in a cold (7–8°C) aerated commercial seawater mixture (Instant Ocean, Aquarium Systems, Wickliffe, Ohio) easily survived 4–6 months without feeding. Fairly large animals, 5–6 cm long when fully contracted, were used throughout.

Binstock and Goldman (1967) reported a 2–3 hr preparation time for *Myxicola* axons. Subsequent modifications in the technique have so greatly reduced the preparation time and increased the frequency of successful preparations that the entire procedure is presented here in some detail. Routinely, between 6 and 7 attempted preparations out of 10 could be successfully brought through this modified dissection procedure, mounted in the recording chamber, have the internal electrodes inserted, and support action potential amplitudes of 100 mv or greater, which is the criterion an axon had to meet to be used in voltage-clamp experiments. In a very few early experiments, animals were first anesthetized by immersion in 5% ethanol in Instant Ocean for 30 min, but for most of the data presented here, unanesthetized animals were used.

To prepare the fibers, a median dorsal incision, just through the body wall, was made for the entire length of the animal, care being taken not to rupture the gut, and the animal was placed, ventral side down on a paraffin dish. Both cut edges of the body wall were pulled aside and pinned to the dish under as much tension as possible without tearing, and the preparation submerged in cold Instant Ocean. A shallow cut, lateral to the gut, along one side for the length of the animal, severed regular connective tissue strands extending over the gut and attached to the body wall. Part of the nerve cord and its dorsal blood vessel were then generally visible under the edge of the gut which lay slightly displaced to one side. The tips of a fine

pair of scissors were inserted between the gut and the nerve cord, and the connective tissue running between the gut and the body wall close to the cord was cut at each segment. If the animal were well-pinned, the gut would fall well to one side, exposing the nerve cord fully. Most of the nerve cord is the giant axon which dominates the dorsal aspect (Nicol, 1948).

A major problem in this preparation is the regular constrictions in the axon, caused by segmental bands of closely apposed connective tissue lying across the cord (Nicol, 1948). These bands may reduce the diameter of the fiber to as little as one-fourth that of the unconstricted regions and so make the insertion of a long internal electrode impossible. Lateral branches of the dorsal blood vessel lie over each connective tissue band and adhere rather tightly to them. Careful segment by segment stripping of the blood vessel, including lateral branches and adhering connective tissue, will, then, relieve these constrictions, producing a more uniform fiber diameter. This stage of the dissection is easily the most critical as it is necessary to work very close to the axon. Nevertheless for the preparation to be useful, care should be taken to insure that the axon is well-opened at each of these sites. Routinely, 3–3.5 cm of axon were prepared in this way. The stripping procedure usually required 15–20 min.

Cuts were made through the body wall, on both sides of the cleaned portion of the cord, and as close to it as could be managed. Best results were obtained in the next stage of the dissection if these cuts closely paralleled the cord. The ends of this cleaned portion (always 3.0 cm in length) were ligated with silk thread and removed to a second chamber. The cord usually lies deeply buried in the body wall. However, to insert the long internal electrode under visual control it is necessary to remove this opaque strip of body wall attached to the cord. To facilitate this procedure, all preparations were stretched to 1.5 times their *in situ* length and anchored at this length by the silk threads. This mounting under tension produced a wider separation between the cord and the strip of body wall and made the axon diameter nearly uniform (although not as uniform as the squid). It was then possible to remove the entire length of body wall in a few seconds by gently stripping it away from the cord with fine forceps. On unstretched cords this procedure might require ¾–1½ hr. Axon diameters after stretch ranged from 425 to 800 μ with a mean, from more than 100 axons, of 560 μ.

The remaining loose muscle and connective tissue could be dissected away fairly readily. Note that this fine cleaning procedure is not strictly analogous to that for squid giant axons, as the *Myxicola* preparation is really the whole nerve cord and not an isolated axon. Fine cleaning may, then, not substantially affect the dimensions of the extracellular space compartment. However, axons were fine cleaned for 2.0–2.5 cm along one side. In this way the axon boundaries could be readily discriminated not only laterally (direct view), but dorsoventrally (mirror view) as well. The entire dissection procedure, including fine cleaning, generally required 40–45 min.

From this stage on, *Myxicola* axons may be treated exactly like squid axons. The recording chamber and reference and chamber electrodes were all as described by Lecar et al. (1967). The recording arrangement and feedback circuit for voltage clamp were as described by Armstrong and Binstock (1964). A few early experiments were done using the internal coaxial electrode (Lecar et al., 1967). For these experi-

ments only those electrodes showing minimal fall-off, as described by these authors, were selected. For most of the experiments, however, the internal current-passing and potential-sensing electrode was the "piggy-back" assembly described by Chandler and Meves (1965). No particular differences in the results obtained by the two electrodes were observed other than that the piggy-back electrode was substantially easier to manufacture. Potential-sensing electrodes in both assemblies contained a floating Pt wire to reduce the high frequency impedance. Resting membrane potentials were corrected for liquid junction potentials using the values of Cole and Moore (1960).

Artificial seawater (ASW) had the following composition: 430 mm Na, 10 mm K, 10 mm Ca, 50 mm Mg, 560 mm Cl, tris(hydroxymethyl) aminomethane 5 mm, pH 8.0. Tetrodotoxin was obtained from Calbiochem (Los Angeles, Calif.). Temperature ranged from 1° to 3°C but never varied by more than 0.5°C during any individual experiment.

RESULTS

Axons for which the initial amplitude of the internally recorded action potential was less than 100 mv were discarded.

Behavior in ASW

The mean initial value of the resting membrane potential was −66.5 mv (58 axons), inside negative, with a range of −54 to −79 mv. These values agree well with the mean value of −69 mv, obtained with a micropipette, reported by Goldman (1968). In Goldman's preparations the axon remained attached to the strip of body wall, and any effect of cut branches on the membrane potential is expected to be small. The close correspondence of the values from the two preparations suggests that cut branches do not in fact present a serious problem for the isolated cord preparation. This might have been expected from the very small size of the branches (Nicol, 1948).

The initial value of the action potential amplitude ranged from the arbitrarily limited minimum of 100 mv to 136 mv with a mean of 112 mv (65 axons). No effort was made to obtain very accurate measurements of the action potential threshold, but the value of the critical depolarization was generally of the order of 15–25 mv. A typical action potential record is included in Fig. 1.

Fig. 1 also shows a series of current records obtained under voltage clamp for a typical axon under these conditions. For depolarizing pulses somewhat above threshold, after a capacitive surge, two components of the membrane current may be recognized: an early, transient inward current and a delayed outward current which rises to a steady state. For stronger depolarizing pulses, the early transient current is first reduced in amplitude then reverses, becoming outward. Properties of the reversal potential are described in the

second paper of this series (Goldman and Binstock, 1969 a). These records are similar to those obtained with other preparations (Cole, 1949; Dodge and Frankenhaeuser, 1958; Julian et al., 1962; Pichon and Boistel, 1967).

Typical current-voltage, $I(V)$, characteristics may be seen in Fig. 3 (open symbols). Values were taken from peak transient (I_p) and steady-state delayed (I_{ss}) currents. The holding potential (zero current reference) was always at the natural resting potential. The transient current displays a negative con-
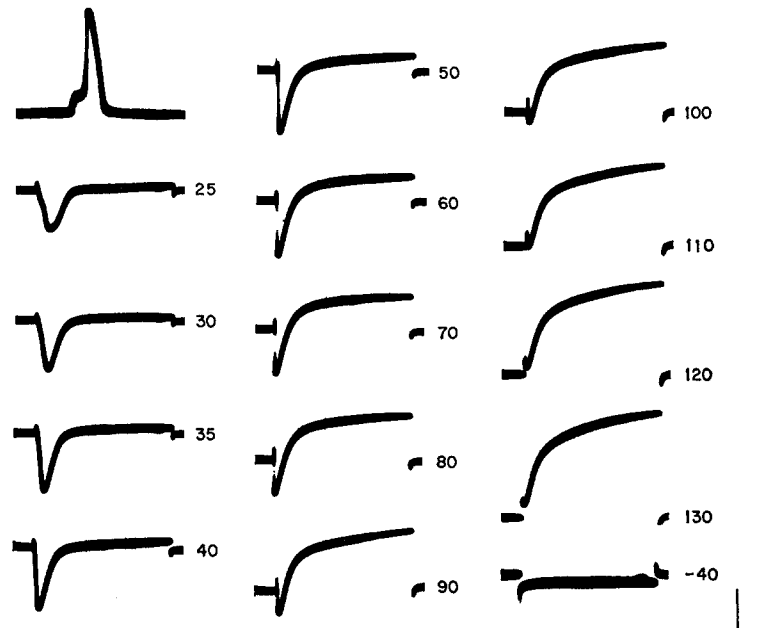

FIGURE 1.   Current- and voltage-clamped records from a *Myxicola* giant axon. The record in the upper left corner is of the action potential. The others are current records under voltage clamp. The numbers beside each current record indicate the displacement in millivolts from the holding potential. Holding potential was −65 mv. Scale, 40 mv, 0.3 ma/cm² for 25 mv command, 0.12 ma/cm² for −40 mv command, 0.6 ma/cm² for all other records, 8 msec.

ductance region. Again, these curves are similar in all essential features to those obtained in squid (Hodgkin et al., 1952), myelinated (Dodge and Frankenhaeuser, 1958), crustacean (Julian et al., 1962), and cockroach (Pichon and Boistel, 1967) axons. Maximum inward I_p's had a mean of 0.87 ma/cm² (29 axons) with a range of 0.41 to 1.66 ma/cm².

All $I(V)$ relations in this series are presented without a leak current correction. This avoids an arbitrary operation on the data. In each case in which $I(V)$ relations under different conditions were compared, the steady-state $I(V)$ curve for hyperpolarizing pulses was substantially the same, never varying by

more than 0.01–0.02 ma/cm² for large hyperpolarizing steps. Fig. 2 shows a typical steady-state $I(V)$ relation obtained for hyperpolarizing steps. Clearly, constant conductance is not a good approximation to the *Myxicola* leak $I(V)$ relation. Rectifying properties of the leak $I(V)$ relation are discussed in the third paper of this series (Goldman and Binstock, 1969 *b*). The mean value of the resting membrane resistance is 1.22 KΩ cm² (27 axons) with a range of 0.31 to 2.33 KΩ cm².

A frequent type of experiment in voltage-clamp studies consists of obtaining a control $I(V)$ relation in ASW, changing the composition of the external medium by substituting some other substance for one of the ionic components, or by adding a drug, again recording an $I(V)$ curve, then returning to ASW and again recording an $I(V)$ curve. It would be useful to have available estimates of what sort of changes in the $I(V)$ relation might be expected from the stress of the clamp measurement and the passage of time alone.

$I(V)$ relations separated by a time interval similar to that needed to effect a complete change of the external medium were obtained from four axons
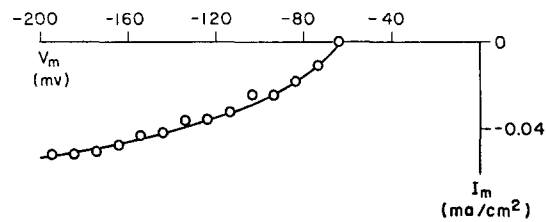

FIGURE 2. Steady-state current observed as a response to hyperpolarizing voltage-clamp steps in a *Myxicola* giant axon.

maintained in ASW. The axon was always removed from potential control during the interval between $I(V)$ runs. Fig. 3 shows the results of one such experiment. The values for the runs at 14 and 29 min agree closely with those for the initial run. These results are summarized in Table I. There is considerable individual variation, but the mean reduction in maximum inward I_p is 7% for run 2 relative to run 1 and 12.7% for run 3 relative to run 1. For I_{ss} at a command pulse of +130 mv the values are 4.3 and 6.9%, respectively. Clearly, treatments producing a reduction in I_p of the order of 10% and in I_{ss} of the order of 5–10%, even when reproducible, cannot be considered as having a demonstrated effect on these current components.

For three axons $I(V)$ runs were continued in ASW over regular time intervals for 3–5 hr. The axons remained extremely vigorous. No effort was made to see how long axons could survive under these conditions. However, the few values we do have indicate that *Myxicola* axons are capable of giving stable quantitative values under voltage clamp for at least 2 and very likely typically in excess of 5 hr. This survival time is adequate for long experiments. These results are also summarized in Table I.
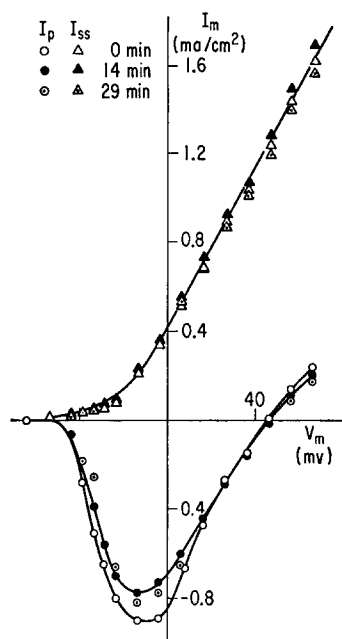
FIGURE 3. Current-voltage relations from a *Myxicola* giant axon in ASW. The initial current-voltage characteristic (open symbols) is compared to that obtained after 14 (solid symbols) and 29 (symbols with dots) min. These time intervals are similar to those needed to effect a complete change of the external medium.

TABLE I

STABILITY OF ELECTRICAL BEHAVIOR
UNDER REPEATED VOLTAGE CLAMP

Axon	Run	Time	Action potential amplitude	Maximum inward I_p reduction*	I_{ss} at +130 reduction*
		min	*mv*	%	%
68M16	1	0	100	—	—
	2	17	100	13.1	14.5
	3	31	90	36.0	16.7
	8	180	65	90.1	41.4
68M74	1	0	110	—	—
	2	14	110	11.1	0
	3	29	110	11.1	3.7
	9	236	110	22.2	5.6
68M75	1	0	114	—	—
	2	14	111	3.6	2.7
	3	28	110	3.6	7.2
	11	300	110	32.5	3.6
68M79	1	0	117	—	—
	2	16	114	0	0
	3	32	114	0	0
Mean	2			7.0	4.3
	3			12.7	6.9

* Always relative to the run 1 value.

Behavior in ASW + Tetrodotoxin

In many preparations tetrodotoxin (TTX) selectively blocks I_p (Narahashi et al., 1964; Nakamura et al., 1965; Hille, 1968; Adrian et al., 1968). However, TTX seems to be ineffective in systems in which I_p is not normally carried by Na (Hagiwara and Nakajima, 1966), and the ability of TTX to block I_p in
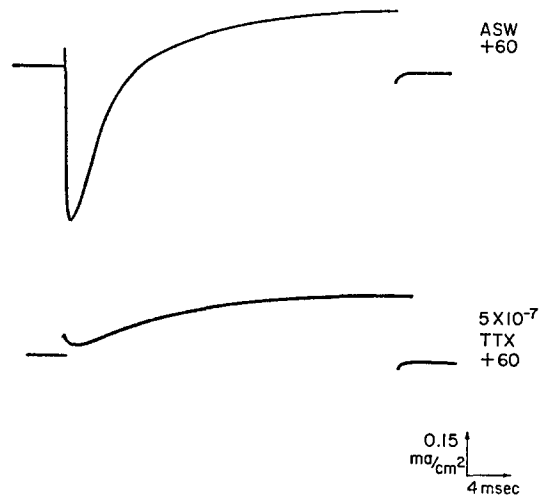

ASW
+60

5×10^{-7}
TTX
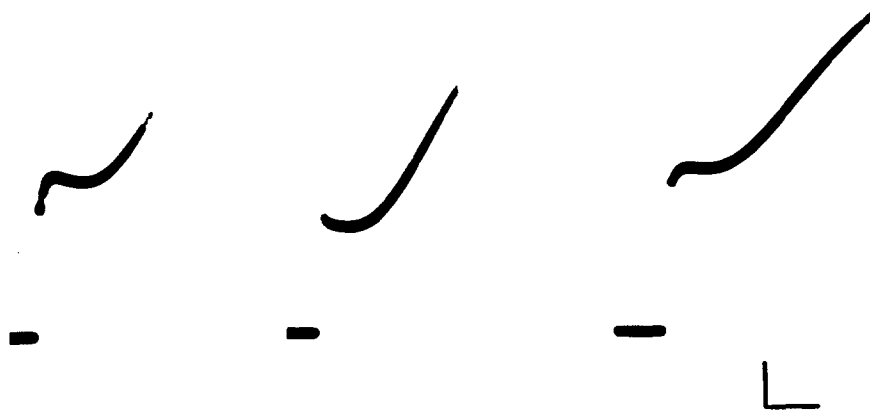+60

0.15
ma/cm^2

4 msec

FIGURE 4 A

FIGURE 4 B

FIGURE 4. A, voltage-clamped current records from a *Myxicola* giant axon in ASW (top) and ASW + 5 × 10^{-7} M TTX (bottom). Both records were obtained at a command pulse of +60 mv. Holding potential and temperature were constant at −68 mv and 1.5°C, respectively. Scale, 0.15 ma/cm^2, 4 msec. B, voltage-clamped current records from a *Myxicola* giant axon in ASW (left), ASW + 1 × 10^{-7} M TTX (center), and again in ASW (right). All records were taken at a command voltage of +160 mv. Temperature, 2°C (left), 1.5°C (center and right). Scale, 0.15 ma/cm^2, 1 msec.

any preparation may of itself be taken as suggesting that Na is the carrier of the I_p.

In *Myxicola* axons TTX at 10^{-7} M completely abolished the spike response, but had no effect on the resting membrane potential. At 5×10^{-8} M a small all-or-none action potential or a graded response persisted. Fig. 4 A shows the effect of 5×10^{-7} M TTX on the voltage-clamp response. At a command pulse of +60 mv, inward I_p is completely blocked while I_{ss} is unchanged. TTX also blocks the outward I_p. Fig. 4 B (left) shows the current record for a depolarizing step, in ASW, large enough to show a clear hump of outward I_p.
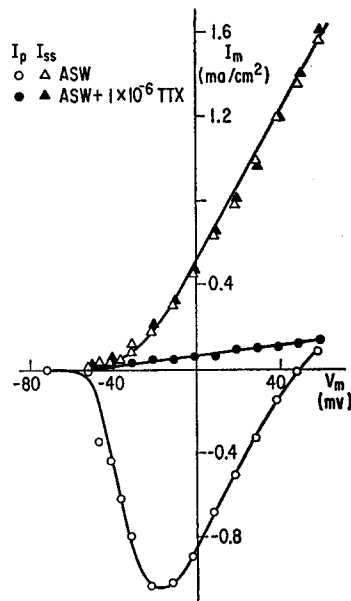

FIGURE 5. Current-voltage relations in a *Myxicola* giant axon. ASW (open symbols). ASW + 1 × 10^{-6} M TTX (solid symbols).

The current and time scales are too expanded to show the development of the I_{ss}. In Fig. 4 B (center), in the presence of 1×10^{-7} TTX, the outward hump of current is abolished, and appears again on return to ASW (Fig. 4 B, right).

Fig. 5 shows the effect of TTX on the $I(V)$ relation. As seen in the current records, I_p is greatly affected, the inward current being completely abolished, while I_{ss} shows very little effect. Recall that these currents are uncorrected for leak. The effects of various doses of TTX on maximum inward I_p and I_{ss} at the same command pulse (+130 mv) are summarized in Table II. The mean value of the reduction in I_{ss} (3.7%) compares well with that obtained from axons kept in ASW only (Table I). These results indicate that for *Myxicola* axons also, TTX selectively blocks I_p.

Note (Table II) that, often, larger reductions in I_{ss} are obtained with smaller TTX doses. This is presumably due to the substantially longer time

required for the action potential to be blocked or to decline to some stable value in low TTX concentrations. As the I_p recovers poorly from TTX at all doses sufficient to block (Table II), we recommend high doses of TTX, 1×10^{-6} M or greater for *Myxicola*, to abolish I_p with minimal effects on I_{ss}.

TABLE II
EFFECT OF TETRODOTOXIN
ON MEMBRANE CURRENTS

Axon	TTX concentration	Maximum inward I_p reduction*	I_{ss} at +130 reduction*	I_p recovery*	Time to block the all-or-none action potential
	M	%	%	%	*min*
68M72	5×10^{-8}	95	0	52.5	—
68M73	5×10^{-8}	99	11	48.5	22.5
68M67	1×10^{-7}	100	6	44.4	14.5
68M68	1×10^{-7}	100	3	50.5	17
68M65	5×10^{-7}	100	13	39.7	9.5
68M66	5×10^{-7}	100	4	23.3	7.5
68M69	1×10^{-6}	100	0	39.4	6
68M71	1×10^{-6}	100	0	56.0	5
68M76	1×10^{-6}	100	0	—	3
68M77	1×10^{-6}	100	0	—	6.5
Mean			3.7	44.3	

* Calculated relative to the initial value in ASW.

DISCUSSION

Myxicola axons are continuously available, reasonably easily prepared, highly reproducible in performance, hardy, stable in behavior, and ready to travel anywhere. They may certainly be expected to be highly useful preparations for the study of membrane processes. In particular, the ready availability means that quantitative reproducibility can be extremely high as experiments may be restricted to only very vigorous axons. Also, investigators may replicate ad lib. rather than on an annual cycle.

Extensive normal values for resting membrane potential, action potential, maximum inward I_p, and resting membrane resistance have been collected. Experience with this preparation has now been sufficient that the data illustrated in Figs. 1 to 3 may be taken as a highly reliable indication of the normal or base line behavior of this preparation in ASW. Axon performance is quite stable in time under voltage clamp. However, reductions in maximum inward I_p of 10% and in I_{ss} of 5–10% are expected in the absence of any particular treatment. Also, our data suggest that cut branches do not present any particular problem in this preparation.

TTX as low as 1×10^{-7} M completely and selectively blocks both the inward

and the outward I_p as well as the action potential itself. There is no effect on the resting membrane potential or I_{ss}. This is taken as suggesting that the early transient current is probably carried by Na.

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